Immunology Letters, 28 (1991) 143–146
Elsevier

IMLET 01571

Sex differences in primary humoral responses of albino rats to human serum albumin

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(Received 10 December 1990; accepted 18 January 1991)

1. Summary

Primary humoral responses to human serum albumin, with or without concomitant Freund's adjuvant, were determined for over 300 male and female Wistar albino rats. Groups of female rats always displayed significantly more antibody production than males. Without adjuvant only 70% of the males responded compared to 99% of the females. When the antigen was accompanied by adjuvant, more than 95% of both sexes responded. Whereas without adjuvant the female response was only twice that of the males, adjuvant facilitated the response to a factor of 10 greater than the males. Both the gender and adjuvant effect each accounted for about 16% of the variance in the measurements of antigen binding capacity. Post-weaning castration did not simulate the gender differences; there were no group differences in ABC at the extremes of estrus-related ambulatory cycles.

2. Introduction

Antibody production is a first line of defense against a myriad of chemical structures that are potentially detrimental to the organism. Individual differences in the capacity to generate appropriate amounts of antibody have obvious relevance to the capacity to accommodate these antigens. Like many other researchers [2], we have found that female albino rats consistently display greater primary humoral responses to a single subcutaneous injection of human serum albumin (HSA) than male albino rats. Our measurement has been antigen binding capacity (ABC); it is expressed in ng/ml of serum and is derived from a modification of the Farr technique. In this paper we present the results of all our experiments from the last six years.

3. Materials and Methods

In the first block of experiments, a total of 333 Wistar albino rats, 60–100 days of age at the time of inoculation, served as subjects; there were 218 males and 115 females. In a total of 8 separate experiments male (n=164) and female (n=54) rats were injected subcutaneously in the right flank with 0.1 ml of 10 μg/ml HSA. Additional groups of males (n=48) and females (n=67) were injected in the flank with a 0.1-ml solution of HSA and 0.1 ml of Freund's complete adjuvant.

In part 1 of the second block of experiments, 18 male Wistar rats were castrated (during brief anesthesia) at between 25–40 days of age; another 18 rats received the same dosage of barbiturate but were not castrated. When the rats were 150 days of age, they were injected (s.c.) in the right flank with 0.1 ml of 10 μg/ml HSA and 0.1 ml of complete Freund's adjuvant. In part 2 of the second block of experiments, 12 100-day-old female rats were injected in the right flank with the usual dosage of HSA (no adjuvant). They were then housed singly in

Key words: Primary humoral response; Human serum albumin; Albino rat; Sex difference

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standard running wheels with food and water available in adjacent caging. Twenty-four hour activity was (numbers of turns/day) recorded so that predictions could be made for each rat's 3 – 5-day cycle. Six of the rats were killed on days of peak activity (estrus) while the other six rats were killed on days during the nadir in activity.

Blood samples for both blocks of experiments were taken by decapitation or from a major tail vein 18 – 20 days after the inoculation and were allowed to clot for 15 min. Serum was separated from the cellular components by centrifugation at $3000 \times g$. The serum was stored at 4 °C until used, usually within a few days. HSA was iodinated with ^{125}I by the lactoperoxidase method according to the procedure provided by the manufacturers of radioiodination reagent (Bio-rad Laboratories, Richmond, CA). The iodinated HSA was separated from unreacted iodine by chromatography over a column of G-25 Sephadex. The iodinated HSA was diluted with cold HSA to give a final concentration of 1.1 μg/ml.

The antigen binding capacity of sera was determined by a modification of a method described by Farr [1]. Test serum or appropriate dilutions of test serum (20 μl), carrier serum from control animals (20 μl), ^{125}I-HSA, 1.1 μg/ml (20 μl) and phosphate buffer, pH 7.2, 0.15 mol/l (60 μl) were incubated for 2 h at 37 °C. Phosphate-buffered saline, pH 7.2 (380 μl) was added, followed by 500 μl of saturated ammonium sulfate, and the mixture was allowed to stand for 15 min at room temperature. The solution was centrifuged for 30 min at room temperature, the supernatant discarded and the precipitate washed with 50% ammonium sulfate (500 μl). The precipitate was then counted with a gamma counter.

The percent of precipitate, corrected for nonspecific binding, for each series of dilutions was plotted on a logarithmic scale against the inverse of the dilution. The dilution required to produce 33% precipitate, in the region of antigen excess, was determined by interpolation. The ABC, expressed in μg of HSA per ml of serum, was calculated according to the formula:

$$\text{ABC} = (\mu g\ ^{125}\text{I-HSA})\ (0.33)\ (1/\text{dilution})\ (50)$$

For those serum samples where antibody levels were not high enough to yield 33% precipitate with neat serum but which yielded more than 10% precipitate, antigen binding capacity was calculated by the formula:

$$\text{ABC} = (\mu g\ ^{125}\text{I-HSA})\ (\%\ \text{precipitate}/100)\ (50)$$

Sera of animals that gave less than 10% precipitate were identified as non-responders and not used in further studies. Our a priori definition of a responder to HSA was a level of at least 0.11 μg/ml of ABC on the day of the baseline sample. Because of the large range of magnitude of the ABC measures, square root transformations were calculated as well. All statistical analyses involved SPSSX software on a Vax 4000 computer.

The means and standard deviations (in parentheses) for the ABC (ng/ml serum) for each of the 4 conditions in the first block of experiments were: male, no adjuvant: 1.1 (1.6); male, adjuvant: 2.9 (4.4); female, no adjuvant: 3.2 (3.2); and female, adjuvant: 24.4 (28.5). Analyses of variance for the magnitude of the primary response as a function of gender (male vs. female) and adjuvant (0.1) demonstrated highly significant sex ($F(1,329)=40.52$, $P<0.001$; $\eta=0.30$) and adjuvant ($F(1,329)=42.94$, $P<0.001$; $\eta=0.31$) effects. The significant ($F(1,329)=36.89$, $P<0.001$) sex × gender interaction was due primarily to the significantly greater response of males that received the adjuvant relative to those who did not.

Homogeneity of variance tests indicated that the variability of ABC measure for females was significantly higher for both the no-adjuvant (Bartlett-Box $=32.01$, $P<0.001$) and adjuvant (Bartlett-Box $=111.09$, $P<0.001$) groups; after square-root transformation, the Bartlett-Box values were reduced to 6.95 and 40.29, respectively. Nonparametric tests (Kruskal-Wallis) for comparison between sexes within the no adjuvant (chi-square $=15.87$, $P<0.001$) and adjuvant (chi-square $=45.03$, $P<0.001$) verified the gender effects. Analyses of the square root transformations display similar sex ($F=98.01$; $\eta=0.42$), adjuvant ($F=81.21$; $\eta=0.39$) and interaction ($F=38.63$) effects.

The percentage of defined responders was: male, no adjuvant 70%; female, no adjuvant 100%; male, adjuvant 96%; and female, adjuvant 100%. Chi-square analyses (33.47; phi-correlation $=0.31$) indicated that the differential sex response to adjuvant was statistically ($P<0.001$) significant. Analyses of

variance for *only* the responders also demonstrated significant sex ($F(1,279)=32.39$), adjuvant ($F(1,279)=36.91$) and interaction ($F(1,279)=30.63$) effects. The means and standard deviations for the 2 male groups after the non-responders had been removed were 1.6 (1.7) and 3.0 (4.4), respectively.

The results of the second block of experiments indicated that post-weaning castration did not simulate ($F(1,34)=0.54$, $P>0.05$) the unual sex differences. The means and standard deviations for the ABC for the rats that were castrated after weaning were 1.7 (1.0) while these values for the controls were 1.3 (1.4). However, analysis of variance indicated that the castrated rats were significantly ($F(1,34)=11.45$, $P<0.001$) lighter than the normal male rats. The means and standard deviations for the ABCs of females during estrus and proestrus were 2.1 (2.0) and 4.5 (5.0), respectively; the differences were not statistically significant ($F(1,10)=1.55$, $P>0.05$).

Our results demonstrate a consistent and significant sex difference in the amount of antibody binding capacity of the Wistar albino rat. Females respond not only more frequently but also with relatively greater antibody production when the antigen is paired with adjuvant. Adolescent castration did not significantly elevate ABC values to within the levels that are typically displayed by normal females. For female rats no conspicuous alterations in ABC occurred when they were killed during their individualized peaks or nadirs in daily activity. Considering the strong coherence of these 3–5-day cycles with estrus, these results suggest a minimum contribution of this hormonal factor to the variability in the primary humoral response.

The failure to elevate the primary response in castrated males in our study suggests that testosterone may not be the mediating factor. McGivern et al. [3] and Persinger and his colleagues [4] have shown that perinatal exposure to extremely low-frequency magnetic fields can produce feminization in male-specific behavior as well as hypertrophy of testicles. Roffi and his group [5] have found that prevention of exposure to endogenous levels of testosterone during the 1–2 h after birth also feminizes male behavior.

Our results suggest that hormonal fluctuations in adult male and female rats are not major determinants of the gender difference in antigen binding capacity. We hypothesize that the source of the gender difference may be related to the sexually dimorphic preoptic hypothalamus; this area is significantly larger in male than female rats. If this idea is correct, then disruption of this structure's development [3, 5] should significantly elevate the primary humoral response in male rats to within normal female levels.

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